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DESCRIPTION

MEG-1 PROTEIN

Technical Field

The present invention belongs to the field of genetic engineering and specifically relates to isolation of a gene of renal cells.

Background Art

Sixty trillion various cells *in vivo* essentially comprise identical genomic DNA. For the normal physiological functions, the expression of these genes is strictly controlled by signals received by cell lines and cells. Therefore, elucidation of genes expressed in each cell type is very important.

A mesangial cell plays a pivotal role in maintaining the structure and function of a glomerulus and also has a central meaning of pathophysiology for each type of nephritis. For example, proliferation of mesangial cells and accumulation of extracellular mesangial matrix are thought to be important pathological finding of glomerulosclerosis in a patient suffering from various glomerular diseases such as chronic nephritis and diabetic nephropathy. Therefore, identification of genes expressed specifically in mesangial cells and elucidation of its function are helpful for understanding biological characteristics of mesangial cells and the causes of diseases relating to mesangial cells, and in turn, treating or diagnosing diseases relating to mesangial cells.

Thy1 antigen is known as a marker for mesangial cells in rats. However, this gene is not specific to mesangial cells and is not expressed in human mesangial cells (Miyata T. et al., Immunology, 1989, 67: 531-533; and Miyata T. et al., Immunology, 1990, 69: 391-395). Mesangial cells are known to express α smooth muscle actin when activated, but this gene is also not specific to mesangial cells. Any genes characteristically in mesangial cells have not been reported.

The present inventor has previously reported MEGSIN as a protein

that is expressed specifically in the mesangial cells (J. Clin. Invest, 1998 Aug 15, 120: 4, 828-36). The present invention relates to a novel protein having a structure that is distinctly different from the MEGSIN.

Disclosure of the Invention

An objective of the present invention is to isolate a gene highly expressed in mesangial cells.

The current inventor isolated mRNA from *in vitro* cultures of human mesangial cells to construct a cDNA library of 3′ side. Then, numerous clones in cDNA library were randomly selected, and determined their sequences. Next, determined sequences were compared with the known nucleotide sequences of cDNA clones of 3′ side obtained from various organs and cells to determine the clones expressed in mesangial cells. Among the clones, one clone that frequently appears in mesangial cells was selected, and its full-length cDNA (3928 bp) was isolated by 5′ RACE method. The whole nucleotide sequence of this full-length cDNA was determined, and thus the present invention was completed. The Kozak translation initiation codon was determined, and then its open reading frame (ORF) was estimated. The protein of this invention having this deduced amino acid sequence was named Meg-1 by the present inventor. The nucleotide sequence of human Meg-1 cDNA and the deduced amino acid sequence for human Meg-1 are shown in SEQ ID NO: 1 and SEQ ID NO: 2, respectively.

An amino acid sequence homology search performed on this amino acid sequence using the SwissProt database confirmed that Meg-1 is a novel protein. Moreover, motif search on the deduced amino acid sequence of Meg-1 was conducted and it revealed a motif that is commonly found in many known protein phosphatases. Since the score of nuclear localization signal is high, it is suggested to be a nuclear protein.

Furthermore, when the topography of Meg-1 was detected by Northern blotting, high expression of Meg-1 was observed in the heart and placenta followed by the expression in the brain, skeletal muscle, kidney, and pancreas. Additionally, weak expression was observed in liver, and expression of Meg-1 was hardly observed in lung.

Characteristically, it was highly expressed in mesangial cells in particular, among primary cultured cells. This invention was completed based on these findings.

This invention specifically relates to the following polynucleotide, protein, and their uses.

(1) A polynucleotide selected from the group consisting of (a) to (d) below:

(a) a polynucleotide comprising a protein-coding region of the nucleotide sequence of SEQ ID NO: 1;

(b) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO: 2;

(c) a polynucleotide comprising the amino acid sequence of SEQ ID NO: 2 in which one or more amino acids are substituted, deleted, inserted and/or added, said polynucleotide encoding a protein functionally equivalent to a protein comprising the amino acid sequence of SEQ ID NO: 2; and

(d) a polynucleotide hybridizing to a DNA comprising the nucleotide sequence of SEQ ID NO: 1 under stringent conditions, said polynucleotide encoding a protein functionally equivalent to a protein comprising the amino acid sequence of SEQ ID NO: 2.

(2) A protein encoded by the polynucleotide of (1).

(3) A vector comprising the polynucleotide of (1).

(4) A transformant carrying the polynucleotide of (1) or the vector of (3).

(5) A method for producing the protein of (2), said method comprising culturing the transformant of (4) and recovering an expression product.

(6) An oligonucleotide having a chain length of at least 15 nucleotides, said oligonucleotide comprising a nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO: 1 or to a complementary strand thereof.

(7) A method for detecting a polynucleotide comprising the nucleotide sequence of SEQ ID NO: 1, said method comprising contacting the oligonucleotide of (6) with a test sample and observing hybridization of said oligonucleotide.

(8) A method for synthesizing a polynucleotide comprising the

nucleotide sequence of SEQ ID NO: 1, said method comprising contacting the oligonucleotide of (6) with a test sample and synthesizing the complementary strand using said oligonucleotide as a primer.

(9) A reagent for detecting mesangial cells, said reagent comprising the oligonucleotide of (6).

(10) A method for detecting mesangial nephritis, said method comprising measuring an expression level of the polynucleotide of (1) in a biological sample, and comparing said level with that obtained from a normal sample.

(11) The method of (10), wherein said biological sample is a mesangial cell.

(12) The method of (10), wherein said expression level is measured using, as an indicator, an mRNA comprising a nucleotide sequence selected from the nucleotide sequence of SEQ ID NO: 1.

(13) The method of (10), wherein said expression level is measured using, as an indicator, the protein of (2) or a fragment thereof.

(14) An antisense polynucleotide against the polynucleotide of (1) or a portion thereof.

(15) An antibody recognizing the protein of (2).

(16) The antibody of (15), recognizing a portion of a protein comprising an amino acid sequence selected from the amino acid sequence of SEQ ID NO: 2.

(17) An immunological assay for measuring the protein of (2) or a fragment thereof based on immunological binding of the antibody of (15) to the protein of (2) or a fragment thereof.

(18) A reagent for immunological assay of the protein of (2) or a fragment thereof, said reagent comprising the antibody of (15).

(19) A transgenic non-human vertebrate, wherein an expression level of a gene encoding Meg-1 is altered.

(20) The transgenic non-human vertebrate of (19), wherein said non-human vertebrate is a mouse.

(21) The transgenic non-human vertebrate of (20), wherein said transgenic non-human vertebrate is a knockout mouse in which the expression of a gene encoding Meg-1 is inhibited.

To fulfill the issues mentioned above, the present inventor used

a 3'-directed cDNA library. This method avoids the variation of cloning efficiency affected by size of DNA. The sequence at the 3' region characterizes each of the genes, and the sequence data of approximately 200 to 300 bp are large enough to demonstrate the characteristics of the gene (Yasuda Y., Miyata T. et al., Kidney Int, 1998 Jan, 53:1, 154-8).

The polynucleotide encoding human Meg-1 of the present invention can be obtained by preparing mRNA from mesangial cells and converting them to the double stranded cDNA by the known methods. mRNA can be prepared by, for example, the guanidine isothiocyanate-cesium chloride method (Chirwin, et al., Biochemistry 18, 5294, 1979), and the treatment with a surfactant and phenol in the presence of deoxyribonuclease (Berger & Birkenmeier, Biochemistry 18, 5143, 1979), etc. Preparation of poly $(A)^+$ RNA from total RNA can be performed by, for example, the affinity chromatography using such a carrier bound to oligo(dT) as Sepharose, cellulose, latex particles, etc. The cDNA (1st strand) can be obtained by treating the above-mentioned obtained mRNA as the template and the oligo (dT), which is complementary to the poly (A) chain at the 3'-terminus, the random primer or the synthetic oligonucleotide, which corresponds to part of the amino acid sequence of Meg-1, as the primer with a reverse transcriptase. The mRNA in the hybrid composed of mRNA and cDNA complementary thereto is partially cleaved with the *E.coli* RNase H. Using the cleaved mRNA as the primer, cDNA (2nd strand) is synthesized by *E.coli* DNA polymerase I. Finally, the double strand cDNA can be obtained by treating with *E.coli* DNA ligase.

The DNA can be cloned by RT-PCR method using poly $(A)^+$ RNA from mesangial cells as a template, and primers synthesized based on the human Meg-1 gene nucleotide sequence. Alternatively, without using PCR, the target cDNA can be obtained by directly screening a cDNA library with a probe synthesized based on human Meg-1 gene nucleotide sequence. The gene of the present invention can be selected by confirming the nucleotide sequence of the gene among the genes obtained by these methods. The Meg-1 cDNA homologues in species other than humans, such as mouse or rat, can be obtained using similar methods.

Otherwise, the Meg-1 cDNA homologues can be isolated as follows. The cDNA encoding the Meg-1 homologue can be isolated by screening the cDNA library by colony hybridization and plaque hybridization, using the nucleotide sequence of the above-mentioned human Meg-1 cDNA as a probe. The cDNA library can be synthesized by using mRNA isolated from mouse or rat tissues, cultured mesangial cells, and the like as template. A commercially available cDNA library (Funakoshi, etc.) may also be used. PCR that utilizes a degenerative primer, which is designed to be placed before and after ORF, based on the cDNA of human Meg-1 of this invention is another possible method that amplifies the cDNA of homologues. A rat homologue of Meg-1 that was obtained based on such a method is disclosed in Examples.

The human Meg-1 genome can be obtained by screening a genomic library. A genomic library can be synthesized, for example, by preparing a genome from human B lymphoblasts and by inserting, into phage vector EMBL3, DNAs partially digested with Sau3 (Blood, vol 83, No 11, 1994: pp 3126-3131). A clone containing the desired genome can be obtained by performing plaque hybridization (see, Shin Saibou Kougaku Jikken (New Cell Biotechnology Experiment) Protocols, Shujun-sha, pp79-92) for such a genomic library. The entire open reading frame region of Meg-1 cDNA (2850 bp), or each of the exon-intron portions obtained by amplifying the human genomic DNA by PCR method using part of the cDNA as primers can be used as probes. A sequence of 5' UTR of the control region sequence can be determined by 5' RACE method (5'-Full RACE Core Set, following Takara's protocol) using human cultured mesangial cell-derived mRNA or human renal mRNA (purchased from Clontech) as a template.

The gene of the present invention can also be produced by following the standard methods using chemical synthesis of nucleic acids, such as phosphoamidite method (Mattencci, M. D. & Caruthers, M. H. J. Am. Chem. Soc. 103, 3185, 1981), phosphite triester method (Hunkapiller, M. et al., Nature 310, 105, 1984).

An eukaryotic gene often shows polymorphism, like human interferon gene, and one or more amino acids may be replaced by this polymorphism with generally maintaining activities of a protein. In general, activities of proteins can be often maintained even if one

or more amino acids are modified. Therefore, polynucleotide encoding a protein obtained by using the artificially modified gene encoding an amino acid sequence of SEQ ID NO: 2 is included in this invention as long as the protein possesses the function typical to the gene of the present invention. The present invention includes protein in which an amino acid sequence of SEQ ID NO: 2 is artificially modified as long as it has characteristics of the proteins of the present invention.

The proteins of the present invention comprise the amino acid sequence of SEQ ID NO: 2 or the amino acid sequence in which one or more amino acids are replaced, deleted, added, and/or inserted, and being functionally equivalent to Meg-1 protein of present invention. In this invention, "functionally equivalent" means having biological properties that are the same as Meg-1. The inventor has found the following biological properties in Meg-1, for example.

First of all, Meg-1 shows, in its amino acid sequence, 20 to 30% homology to protein phosphatase 2A (PP2A) subunit A (regulatory subunit) from a variety of species including human. PP2A is known to form a trimer in which subunit C (a catalytic subunit having enzyme activity) is bound to the C terminal region of subunit A and subunit B (another regulatory subunit specific for tissues and cells) to the N terminal region of subunit A. While subunits A and C have a common structure among the tissues and cells, subunit B is thought to have a structure specific for tissues and cells. Based on this information, subunit B having, as subunit A, Meg-1 of the present invention that shows homology to PP2A has the possibility of a mesangial cell-specific protein. Subunit B having Meg-1 as subunit A is thought to be involved in a mechanism for regulating the mesangial cell-specific activity.

The characteristic feature of the Meg-1 expression was a particularly high level of expression in the primary culture of mesangial cells. In other primary cell cultures, its expression was observed in dermal fibroblasts and renal cortical epithelial cells, and also in endothelial cells of umbilical vein and smooth muscle cells at trace levels. When the expression in the different tissues were compared, abundant expression of Meg-1 was observed in heart

and placenta, and then in brain, skeletal muscle, kidney and pancreas. In addition, low-level expression of the gene was observed in liver, but no expression in lung. Among the tumor cell lines, high level expression of the gene was observed in HeLa cell line S3, chronic myelocytic leukemic cell line K-562, lymphoblastic leukemia cell line MOLT-4, colon adenocarcinoma cell line SW480 and lung carcinoma cell line A549. Also, promyelocytic leukemia cell line HL-60 and melanoma cell line G361 were observed to express the gene. Expression in Burkitt's lymphoma Raji was almost negligible. Analysis of the rat Meg-1 expression in the rats showed that Meg-1 was highly expressed in the kidney (particularly in mesangial cells) both in humans and rats, suggesting the possibility that Meg-1 is one of the functional genes responsible for mesangial cell function. The state of Meg-1 expression in each tissue and cultured cell can be investigated by Northern blotting assay using mRNA prepared from each tissue as a sample, with a probe having, for example, a sequence selected from the nucleotide sequence of SEQ ID NO: 1.

Any proteins functionally equivalent to Meg-1 for the biological features as described above will constitute Meg-1 of the present invention. Thus, the present invention includes not only human Meg-1, whose structure has been specifically elucidated, but also its homologues derived from other species, which have equivalent structure and functions.

Among the known amino acid sequences verified by homology search, $PP4_{R1}$ (J.Biol.Chem.274(9), 5339-5347, 1999) shows the closest structural similarity with Meg-1. However, $PP4_{R1}$ is a protein that has been reported to be a human homologue of a regulatory subunit of protein serine/threonine phosphatase 4 isolated from the bovine small intestine. In their structures, the serine residue located at position 18 from the N-terminus of $PP4_{R1}$ is replaced with 18-amino acid residues of FGVDDYSSESDVIIIPSA in Meg-1. Thus, the two proteins are different in the structure. Also, from a functional point of view, they are different, because $PP4_{R1}$ was isolated from neuroepithelial cells, based on the amino acid sequence of a protein from bovine small intestine, but in contrast, Meg-1 is strongly expressed in mesangial cells. Since the two proteins have continuous amino acid sequences

different from each other, it is possible that both Meg-1 and $PP4_{R1}$ are isoforms encoded by a plurality of genes or that they are splicing variants.

The polynucleotide of the present invention includes polynucleotides encoding these functionally equivalent proteins. The polynucleotides encoding these proteins can be cDNA, genomic DNA, synthetic DNA, RNA, and artificial polynucleotide molecule constituted by nucleotide derivatives.

The codons for desired amino acids themselves are well-known, can be optionally selected, and can be determined by following the standard method by, for example, considering the frequency of use of codons in hosts to be used (Grantham, R. et al. Nucleic Acids Res. 9, r43, 1981). Therefore, the present invention includes polynucleotides appropriately modified by degeneration of codons. It is possible to partially change the codons of these nucleotide sequences by following site-specific mutagenesis methods (Mark, D. F. et al., Proc. Natl. Acad. Sci. U.S.A. 1984, 81: 5662) and such that uses chemically synthesized oligonucleotides encoding the desired modifications as primers.

A polynucleotide hybridizing with a polynucleotide containing the nucleotide sequence of SEQ ID NO: 1 and encoding a protein that typically functions as Meg-1 of the present invention, can be included in the polynucleotides of the present invention. A sequence capable of hybridizing with specific sequences under stringent conditions is thought to have the activities similar to a protein encoded by the specific sequences. Stringent conditions generally indicate the following conditions. That is, hybridization is carried out at 65°C in 4X SSC, and then washing is carried out for one hour at 65°C using 0.1X SSC. The temperature of hybridization and washing, which greatly affect stringency, can be adjusted depending on the melting temperature (Tm). Tm changes, depending on the proportion of constituent bases occupying the base pairs to be hybridized, and by the composition of the hybridization solution (salt concentration, concentrations of formamide and sodium dodecyl sulfate). Therefore, through experience, one skilled in the art can consider these conditions mentioned above to set appropriate conditions that will

yield similar stringency.

The nucleotide sequences of polynucleotides of the present invention, including mutants, can be used for various purposes based on known techniques.

Other prokaryotic or eukaryotic hosts can be transformed by inserting the polynucleotide encoding Meg-1 cloned as described above into an appropriate expression vector DNA. Moreover, the gene can be expressed in each host cell by introducing an appropriate promoter and sequences relating to the phenotypic expression into the expression vector. As an expression vector, for example, pET-3 (Studier & Moffatt, J. Mol. Biol. 189, 113, 1986) and such for *E. coli*, pEF-BOS (Nucleic Acids Research 18, 5322, 1990), pSV2-gpt (Mulligan & Berg, Proc. Natl. Acad. Sci. U. S. A. 78, 2072, 1981), and so on for COS cells, and pVY1 (WO89/03874) and such for CHO cells can be used. The target proteins can be expressed as a fusion protein derived from a fusion gene between a target gene and a gene encoding other polypeptide. Such fusion proteins can easily be purified and separated to isolate a desired protein. Histidine tag, c-myc tag, MBP-tag, GST-tag, and the like are known as proteins to be fused. Vectors that can express inserts in which these tags are fused are commercially available.

For example, *Escherichia coli* can be used as prokaryotic host cells in the expression system of the present invention. *Saccharomyces cerevisiae* and such can be used as microbiological host cells among eukaryotic organisms. Examples of mammalian host cells include COS cells, CHO cells, BHK cells, etc. The transformants of the current invention can be cultured under appropriately selected culturing condition suitable for host cells.

As mentioned above, Meg-1 can be produced by culturing the transformants transformed with the polynucleotide encoding the target Meg-1, and recovering it from the cells or the culture supernatant. It can be purified into a substantially pure protein. Meg-1, a target protein of the present invention, can be separated and purified by the separation and purification methods commonly used for proteins, and the method is not particularly limited. Meg-1 can be separated and purified by, for example, appropriately selecting and combining

various chromatographies.

Besides the methods descried above, the gene of the present invention, the recombinant vector comprising the gene, the transformants carrying the vector, and the production of Meg-1 using gene manipulation can be manipulated by the standard method described in "Molecular Cloning - A Laboratory Manual" (Cold Spring Harbor Laboratory, N. Y.).

In addition, a probe for detecting a Meg-1 gene can be designed based on the nucleotide sequence of SEQ ID NO: 1. Moreover, primers for amplifying DNA and RNA containing these nucleotide sequences can be designed. It is routine for a person skilled in the art to design probes and primers based on a given sequence. An oligonucleotide comprising a designed nucleotide sequence can be chemically synthesized. These oligonucleotides can be used for the hybridization assay of various formats, or for the synthetic reaction of nucleic acids, such as PCR, if appropriately labeled. An oligonucleotide used as a probe or a primer has at least 15 bases, and preferably 25 to 50 bases. Probes (or primers) that can distinguish Meg-1 and $PP4_{R1}$ can be prepared by designing them to contain a nucleotide sequence selected from a part corresponding to the 18 amino acid-encoding region (74-127) that is structurally different to $PP4_{R1}$, which shares almost same nucleotide sequence.

The present invention provides a polynucleotide comprising at least 15 nucleotides and complementary to a polynucleotide comprising the nucleotide sequence of SEQ ID NO: 1 or its complementary strand. Herein, the term "complementary strand" is defined as one strand of a double strand polynucleotide composed of A:T (in RNA, T is replaced to read U) and G:C base pair to the other strand. Also, "complementary" is defined as not only those completely matching within a continuous region of at least 15 nucleotides, but also having a homology of at least 70%, favorably 80%, more favorably 90%, and most favorably 95% or higher within that region. The homology may be determined using the algorithm described herein. Since Meg-1 is highly expressed in mesangial cells, probes and primers for detecting the Meg-1 gene of this invention can be used to identify and detect the mesangial cells. Primers are especially useful to amplify the

Meg-1 gene. If necessary, labels or binding tags can be added to the oligonucleotides of the invention.

The present invention also provides a method for detecting mesangial nephritis using Meg-1 expression as an indicator. Expression of the polynucleotide of the present invention is increased in the mesangial cells of the kidney tissue in which mesangial nephritis has been developed. This is supported by the observation obtained in the working example that rat Meg-1 mRNA was strongly expressed in the rat mesangial cells in which mesangial nephritis was induced by administration of anti-Thy1 monoclonal antibody. Therefore, mesangial nephritis can be detected when Meg-1 expression is increased, by measuring Meg-1 expression in a biological sample and comparing the measurement with that obtained from a normal sample. Mesangial cells, urine and blood may be used as the biological samples. Meg-1 expression levels may be compared by measuring mRNA or its translational product Meg-1 protein, or fragments thereof. Methods for measuring these components in biological samples are well known. For example, Meg-1 mRNA may be detected or measured by Northern blotting or RT-PCR. Meg-1 protein or a fragment thereof may be detected using an antibody recognizing Meg-1. Where mesangial cells are used as a biological sample, cellular localization of these components can be elucidated using *in situ* hybridization or immunohistological detection.

Furthermore, an antisense polynucleotide that may regulate the expression of Meg-1 is provided based on the nucleotide sequence of the gene encoding Meg-1 disclosed in this invention. The antisense polynucleotide of this invention is an important tool for demonstrating the role of Meg-1 in the mesangial cells. It is also useful for regulating diseased conditions caused by accelerated expression of Meg-1. The effects of inhibiting the expression of target genes by antisense polynucleotides are the following. They are inhibition of transcription initiation by triple strand formation, transcription inhibition by hybrid formation with a site that has formed a local open loop structure due to RNA polymerase, transcription inhibition by hybrid formation with RNA that is being synthesized, splicing inhibition by hybrid formation at the joint

between intron and exon, splicing inhibition by hybrid formation with the spliceosome forming region, inhibition of mRNA transition from the nucleus to the cytoplasm by hybrid formation with mRNA, splicing inhibition by hybrid formation with the capping region and the poly(A) attached region, inhibition of translation initiation by hybrid formation with the translation initiation factor binding region, translation inhibition by hybrid formation with the ribosome binding region near the initiation codon, interception of protein chain elongation by hybrid formation with the translation region of mRNA and polysome binding region, and inhibition of gene expression by hybrid formation with the nucleic acid-protein interacting region, and so on. These inhibit the expression of the target gene by inhibiting the transcription, splicing, or translation processes (Hirashima and Inoue, Shin Seikagaku Jikken Koza 2 Kakusan IV Idenshi no Fukusei to Hatsugen (New Biochemistry Laboratory Experiment 2 Nucleic Acids IV Replication and Expression of Genes), Japanese Biochemical Society Edition, Tokyo Kagaku Dojin, pp. 319-347, 1993).

The antisense sequence used in this invention may inhibit the expression of the target genes by any one of the effects mentioned above. In one embodiment, if an antisense sequence complementary to the non-translational region located near the 5′ end of mRNA of the gene is designed, it may be effective for inhibiting the translation of the gene. However, sequences complementary to the coding region or to the 3′end non-translational region may also be used. In this manner, the antisense polynucleotide utilized in this invention includes polynucleotide containing the antisense sequence of not only the translational region of the gene, but also the sequence of the non-translational region. The antisense polynucleotide to be utilized is inserted downstream of the appropriate promoter, and preferably, a sequence including a transcription-terminating signal is inserted to the 3′ end. DNA prepared in this manner can be transformed into the desired host by known methods. Preferably, the antisense polynucleotide sequence should contain a sequence that is complementary to the endogenous gene (or its homologous genes) of the host to be transformed or its portion. However, as long as gene expression is inhibited effectively, complete complementarity is not

necessary.

The RNA that is transcribed using antisense polynucleotide as template is designed to have preferably 90% and most preferably 95% complementarity to the transcription product of the target gene. The length of the antisense polynucleotide used for effectively inhibiting the expression of target genes is at least 15 nucleotides or more, preferably 100 nucleotides or more, and more preferably 500 nucleotides or more. Usually, the length of the antisense RNA used is shorter than 2.5 kb.

A promoter region and an enhancer region of Meg-1 gene existing in genome can be obtained based on the cDNA nucleotide sequence of Meg-1 of the present invention. Specifically, these control regions can be obtained by the same method as described in unexamined published Japanese patent application (JP-A) No. Hei 6-181767; The Journal of Immunology, 1995, 155, 2477-2486; Proc. Natl. Acad. Sci. USA, 1995, 92, 3561-3565; etc. Herein, a promoter region means DNA region existing upstream of a transcription initiation site to control the expression of a gene, and an enhancer region means DNA region existing in an intron or 3'UTR to control expression of a gene.

Specifically, a promoter region can be obtained, for example, by the following method.

1) A promoter region of Meg-1 is cloned from a human genomic library using 5' end site of cDNA of Meg-1 as a probe.

2) Meg-1 gene is digested with restriction enzyme to obtain a DNA comprising the promoter region at the upstream region (2 to 5 kbp) containing a translation initiation codon of Meg-1 gene and determine the nucleotide sequence. The transcription initiation site (+1) is determined using poly(A)$^+$RNA prepared from human mesangial cells as a template, by the primer elongation method using primer DNA selected from cDNA sequence at 5' end site of Meg-1 gene. A site possibly comprising the promoter activity is predicted by searching transcription factor binding sequence from the nucleotide sequence.

3) The DNA fragment excluding the coding region of Meg-1 gene from the DNA obtained in 2) is subcloned in a plasmid, and a chloramphenicol acetyl transferase (CAT) gene or a luciferase gene

is ligated as a reporter gene at 2 to 5 kbp downstream of the DNA fragment to construct a reporter plasmid. Similarly, DNA fragments corresponding to various sites upstream of Meg-1 gene, in which 5′ and 3′ end sites are stepwise removed, are prepared by digestion with restriction enzymes or by PCR to include possible promoter regions. The CAT gene or the luciferase gene is ligated as a reporter gene at downstream of these DNA fragments to construct a reporter plasmid.

4) A promoter region upstream of Meg-1 gene is obtained by measuring CAT or luciferase activity in animal cells transformed with the reporter plasmid prepared in 3).

A 3′ UTR and an enhancer region in introns can be obtained by cloning genomic genes of human Meg-1 from a human genomic library using Meg-1 cDNA as a probe in the same manner as described above for the promoter.

Transcription factors controlling the expression of Meg-1 gene can be obtained by the known methods, for example, those described in "Shin Saibou Kougaku Jikken (New Cell Biotechnology Experiment) Protocols, Shujun-sha," "Biomanual series 5 Tensha Inshi Kenkyu-hou (studies on transcription factors), Yodo-sha," "DNA & Cell Biology, 13, 731-742, 1994," such as affinity chromatography, South-western method, footprinting method, gel shift method, or one-hybrid method. Herein, a transcription factor means a factor controlling the transcription of Meg-1 gene, including a transcription initiation factor that induces the transcription initiation reaction and a transcription control factor that up- or down-regulates transcription.

Affinity chromatography can be performed by applying a nucleic extract to an affinity column in which promoter and enhancer regions obtained above are immobilized on Sepharose or latex beads, washing the column, eluting the binding transcription factor using a DNA comprising the same sequence as that immobilized in the column, and recovering the transcription factor controlling the expression of Meg-1 gene.

In case where the South-Western method is employed, the expression product of the cDNA inserted in the *E.coli* expression vector (e.g. λgt11) is screened as per the tagged-probe. For example,

the cDNA to be screened is expressed as a fusion protein with the β-galactosidase, and is adsorbed to the nitrocellulose filter. Then, the transcription factor, which regulates the Meg-1 gene expression, can be obtained by selecting those phages that synthesize the fusion protein with a binding activity using radioisotope tagged DNA fragments of the promoter region or enhancer region as the probe.

The gel shift method is based on the phenomenon that electrophoretic mobility on a polyacrylamide gel of DNA changes when it is bound to a protein. DNA fragments of the promoter region and enhancer region are used as probes and upon mixing with samples containing transcription factors (for example, nuclear protein extract), they are analyzed by electrophoresis under low ionic strength. The binding of transcription factors is detected as a band with mobility different from that of free DNA. The gel shift method allows separation of transcription factors from a mixture of proteins with high sensitivity.

Upon further analysis by the footprint method, the DNA-transcription factor complex obtained by the gel shift method allows determination of the transcription factor-binding site. The footprint method utilizes the phenomenon that when a protein binds to DNA, it is protected from DNase I digestion. That is, DNA of the promoter region and enhancer region labeled at the end with ^{32}P is partially digested by DNase I in the presence of transcription factors, and then separated by degenerate polyacrylamide gel used for determining nucleotide sequences. Comparison to the result when the same treatment is carried out in the absence of a transcription factor shows the disappearance of a band due to transcription factor binding, and therefore, estimation of the binding region becomes possible.

The present invention also provides an antibody recognizing Meg-1. The antibody of the present invention includes, for example, an antibody against the protein comprising the amino acid sequence of SEQ ID NO: 2. An antibody (for example, a polyclonal antibody, a monoclonal antibody) or an antiserum against Meg-1 or a partial peptide of Meg-1 of the present invention can be produced by a known method for producing an antibody and antiserum, using Meg-1 of the present invention, a partial peptide of Meg-1 of the present invention,

or a fusion protein such as c-myc-$(His)_6$-Tag-Meg-1 or MBP-Meg-1 of the present invention as a antigen.

The Meg-1 of the present invention or a partial peptide of Meg-1 of the present invention is administered with well-known carrier or diluent to a warm-blooded animal at the site capable of producing an antibody. To enhance the antibody productivity, the complete Freund's adjuvant or incomplete Freund's adjuvant can be administered together with the antigen. Immunization is performed every one to six weeks, a total of about 2 to 10 times, in general. Warm-blooded animals to be used are, for example, a monkey, rabbit, dog, guinea pig, mouse, rat, sheep, goat, and chicken, and preferably a mouse and rabbit.

Monoclonal antibody-producing cells can be prepared by selecting immunized warm-blooded animals, such as mice, in which an antibody titer is detected, obtaining spleen or lymph node from the animals 2 to 5 days after the final immunization, and fusing the antibody producing cells contained in these tissues with myeloma cells to obtain monoclonal antibody-producing hybridoma. The antibody titer in antiserum can be measured by, for example, reacting the labeled Meg-1 described below with antiserum, and measuring an activity of the label binding to the antibody. Cell fusion can be performed by a known method, for example, the method of Kohler and Milstein (Nature, 256, 495, 1975). Polyethylene glycol (PEG), Sendai virus, and such can be used as a fusion enhancer, and PEG is preferable.

Examples of myeloma cells include X-63Ag8, NS-1, P3U1, SP2/0, AP-1, and such, and X-63Ag8 is preferably used. The ratio of the number of antibody-producing cells (splenic cells) to that of myeloma cells is 1:20 to 20:1. Cells can be fused efficiently by adding PEG (preferably PEG1000 to PEG6000) at the concentration of about 10 to 80%, and incubating for 1 to 10 min at 20 to 40°C, preferably at 30 to 37°C. Anti-Meg-1 antibody-producing hybridoma can be screened by various methods, for example, the method in which the hybridoma culture supernatant is added to a solid phase (for example, a microplate) on which Meg-1 antigen is adsorbed directly or with a carrier, and anti-immunoglobulin antibody labeled with a radioactive substance, an enzyme, and such (When cells used for cell fusion are

derived from a mouse, anti-mouse immunoglobulin antibody is used.) or protein A is added thereto, and anti-Meg-1 monoclonal antibody binding to the solid phase is detected, the method in which the hybridoma culture supernatant is added to a solid phase on which anti-immunoglobulin antibody or protein A is adsorbed, and Meg-1 labeled with a radioactive substance, an enzyme, and such is added thereto, and anti-Meg-1 monoclonal antibody binding to the solid phase is detected.

Anti-Meg-1 monoclonal antibody can be selected and cloned by known methods or modified methods thereof using usually a culture medium for animal cells supplemented with HAT (hypoxanthine, aminopterin, and thymidine). Any medium for selection, cloning, and culturing can be used as long as hybridoma can grow therein. For example, RPMI 1640 medium (Dainippon Pharmaceutical) containing 1 to 20%, preferably 10 to 20% of fetal bovine serum, GIT medium (Wako Pure Chemicals) containing 1 to 10% fetal bovine serum, or serum-free medium for hybridoma culturing (SFM-101, Nissui Pharmaceutical) can be used. Incubation temperature is generally 20 to 40°C, preferably about 37°C. Incubation time is generally 5 days to 3 weeks and preferably 1 to 2 weeks. Incubation is performed under the 5% carbon dioxide gas in general. The antibody titer of the hybridoma culture supernatant can be determined in the same manner as described above for the measurement of anti-Meg-1 antibody titer in the antiserum. Cloning can be generally conducted by known methods, for example, semisolid agar method, or limiting dilution method. A cloned hybridoma is cultured preferably in a serum-free medium, thereby producing an optimal amount of an antibody in the supernatant. A target monoclonal antibody can be obtained in ascites.

A monoclonal antibody of the present invention does not crossreact with other proteins other than Meg-1 by selecting those capable of recognizing epitopes specific to Meg-1. In general, an epitope specific to a protein is composed of at least 7 or more continuous amino acid residues, preferably 10 to 20 amino acids in an amino acid sequence of the protein. Therefore, a monoclonal antibody recognizing an epitope composed of peptides having an amino acid sequence selected from the amino acid sequence of SEQ ID NO:

2 and composed of at least 7 continuous amino acid residues can be the monoclonal antibody specific to Meg-1 of the present invention. More specifically, for example, it can be said that an antibody recognizing the 18 amino acid-comprising region (18 to 35th residues from the N-terminus) that is structurally different to $PP4_{R1}$, and distinguishing structural differences with a highly homologous protein such as $PP4_{R1}$, is a preferable embodiment of the antibody recognizing Meg-1 of this invention.

An anti-Meg-1 monoclonal antibody can be separated and purified by the separation and purification method of immunoglobulin commonly used for the separation and purification of polyclonal antibodies. The known purification methods include, for example, salting out, alcohol precipitation, isoelectric point precipitation, electrophoresis, adsorption and desorption method by ion exchanger (for example, DEAE), ultra centrifugation, gel filtration, or specific purification method whereby antibody is exclusively collected by, for example, an antigen binding solid phase or active adsorbent, such as Protein A or Protein G, and the binding is dissociated to obtain the antibody.

Monoclonal antibodies and polyclonal antibodies recognizing Meg-1 of the present invention, obtained in such a manner, can be used for the diagnosis and treatment for diseases relating to mesangial cells. These antibodies can be used as a tool for identifying or detecting mesangial cells because Meg-1 is a protein that is highly expressed in mesangial cells. Methods for immunologically detecting cellular proteins are commonly known. Examples of a method for measuring Meg-1 with these antibodies include an sandwich assay comprising reacting Meg-1 with an antibody binding to an insoluble carrier and a labeled antibody and detecting Meg-1 in the sandwiched complex produced by the reaction, or a competition method comprising competitively reacting labeled human Meg-1 and human Meg-1 in a sample with an antibody to measure human Meg-1 in a samples based on labeled antigen amount reacted with the antibody.

The measurement of human Meg-1 by the sandwich method is conducted by, for example, the 2 step method in which an immobilized antibody is reacted with Meg-1, unreacted materials are completely

removed by washing, and a labeled antibody is added to form a complex of the immobilized antibody Meg-1 the labeled antibody, or one step method in which the immobilized antibody, the labeled antibody, and Meg-1 are mixed at the same time.

Examples of an insoluble carrier used for the measurement include, for example, polystyrene, polyethylene, polypropylene, polyvinyl chloride, polyester, polyacrylate, nylon, polyacetal, synthetic resin such as fluoride resin, etc., polysaccharides such as cellulose, agarose, and such, glass, metals, etc. The form of an insoluble carrier can be varied and includes tray, spheroid, particle, fiber, stick, board, container, cell, test tube, etc. The antibody-adsorbed carrier should be stored at a cool place in the presence of appropriate preservatives, such as sodium azide.

Antibodies can be immobilized by known chemical binding or physical adsorption methods. Chemical binding methods include, for example, a method using glutaraldehyde, the maleimide method using N-succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate, N-succinimidyl-2-maleimidoacetate, and such, and the carbodiimide method using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, etc. In addition, the maleimidobenzoyl-N-hydroxysuccinimidoester method, the N-succimidyl-3-(2-pyridyldithio)propionate method, the bisdiazolated benzidine method, and dipalmityllysine method. Alternatively, the complex produced by reacting two different antibodies against a substance to be detected and an epitope is captured with the third antibody immobilized by the above method.

Any label useful for immunoassay can be used without being limited. Specifically, enzymes, fluorescent substances, luminescent substances, radioactive substances, metal chelates, and such, can be used. Preferable labeling enzymes are, for example, peroxidase, alkaline phosphatase, β-D-galactosidase, malate dehydrogenase, *Staphylococcus* nuclease, delta-5-steroid isomerase, α-glycerol phosphate dehydrogenase, triosephosphate isomerase, horseradish peroxidase, asparaginase, glucose oxidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase, and acetylcholine esterase, etc. Preferable fluorescent substances

include, for example, fluorescein isothiocyanate, phycobiliprotein, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, and orthophthalaldehyde. Preferable luminescent substances include, for example, isoluminol, lucigenin, luminol, aromatic acridiniumester, imidazole, acridinium salt and its modified ester, luciferin, luciferase, and aequorine. Preferable radioactive substances include, for example, ^{125}I, ^{127}I, ^{131}I, ^{14}C, ^{3}H, ^{32}P, ^{35}S, etc.

The method for binding the above labels is known. Specifically, direct and indirect labeling can be used. The common direct labeling is the method in which an antibody or an antibody fragment is chemically covalent-bound with a label using a crosslinking agent. Crosslinking agents include N,N'-orthophenylenedimaleimide, 4-(N-maleimidomethyl) cyclohexanoate N-succinimide ester, 6-maleimidohexanoate N-succinimide ester, 4,4'-dithiopyridine, and other known crosslinking agents. The crosslinking agent can be reacted with enzymes and antibodies by the known methods depending on the characteristics of the crosslinking agent. An example of the indirect labeling method comprises binding an antibody to a low molecular weight hapten such as biotin, dinitrophenyl, pyridoxal, or fluorescamine, and indirectly labeling the antibody with the binding partner to the hapten. Avidin and streptoavidin can be used as a recognition ligand for biotin, whereas dinitrophenyl, pyridoxal, or fluorescamine are labeled with antibodies recognizing these haptens. Horseradish peroxidase can be used as an enzyme for labeling antibodies. This enzyme is useful because it can react with many substrates and be easily bound to antibodies by the periodate method. Occasionally, as an antibody, their fragments, for example, Fab', Fab, $F(ab')_2$ are used. Both polyclonal and monoclonal antibodies can be labeled with an enzyme by the same method. Enzyme-labeled antibodies obtained using the above crosslinking agent can be purified by the known methods such as affinity chromatography, and such, to serve in a more sensitive immunoassay system. Purified enzyme-labeled antibodies are stored with a preservative such as thimerosal and a stabilizer such as glycerol. Labeled antibodies can be lyophilized and stored in the cool and dark place for a long time.

When a label is an enzyme, its substrate and, if necessary, a

coloring agent is used for measuring its activity. When peroxidase is used as an enzyme, H_2O_2 is used as a substrate solution and 2,2'-azino-di-[3-ethylbenzothiazolinesulfonic acid] ammonium salt (ABTS), 5-aminosalicylic acid, orthtophenylenediamine, 4-aminoantipyrine, or 3,3',5,5'-tetramethylbenzidine, and such, is used as a coloring agent. When alkaline phosphatase is used as an enzyme, orthonitrophenylphosphate, paranitrophenylphosphate, and such, can be used as substrates. When β-D-galactosidase is used as an enzyme, fluorescein-di-(β-D-galactopyranoside), 4-methylumbelliferyl-β-D-galactopyranoside, and such, can be used as substrates. The present invention also includes an immunoassay reagent for Meg-1, comprising labeled or immobilized monoclonal or polyclonal antibodies, and further includes a kit comprising this reagent and an indicator for detection label and a control sample, etc.

Any biological samples such as body fluid such as blood plasma, serum, blood, urine, tissue fluid, or cerebrospinal fluid and such can be used as samples for measuring the Meg-1 of the present invention as long as they contain Meg-1 or its precursor or a fragment.

In addition, the present invention relates to a transgenic nonhuman vertebrate in which the expression level of Meg-1 gene is altered. Herein, Meg-1 gene includes cDNA, genomic DNA, or synthetic DNA encoding Meg-1. Gene expression includes both steps of transcription and translation. Transgenic animals of the present invention are useful for investigating function and expression control of Meg-1, clarifying mechanisms of development of diseases relating to human mesangial cells, and developing disease model animals used for screening and testing safety of pharmaceuticals.

In the present invention, Meg-1 gene can be modified so as to artificially increase or decrease its expression level compared with the original gene by introducing mutation such as deletion, substitution, insertion, and such, in a part of some important sites (enhancer, promoter, intron, etc.) which control the normal expression of Meg-1 gene. Such modification alters transcription of Meg-1 gene. On the other hand, translation to proteins can be modified by deleting a part of an exon, or replacing a certain codon with a

stop codon by introducing point mutation into coding regions. Such mutation can be introduced by the known methods for obtaining transgenic animals.

Transgenic animals means, in a narrow sense, animals into reproductive cells of which an exogenous gene is artificially introduced by genetic recombination, and in a broad sense, animals into chromosome of which an exogenous gene is stably introduced during an early developmental stage, the gene can be transmitted to the offspring as genotype, including antisense transgenic animals in which the function of a specific gene is inhibited by antisense RNA, animals in which a specific gene is knocked out by using embryonic stem cells (ES cells), and animals into which point mutation DNA is introduced. Transgenic animals used herein include all vertebrates except for human.

Transgenic animals can be prepared by the method comprising mixing a gene with an egg and treating the mixture with calcium phosphate, the microinjection method whereby a gene is directly injected into a nucleus in pronuclear egg by a micropipette under the phase contrast microscope (microinjection method, U.S. Patent No. 4,873,191), and the method using embryo stem cells (ES cells). Other methods include, for example, the method in which a gene is inserted into a retrovirus vector to infect an egg and the sperm vector method in which a gene is introduced into an egg through sperm, etc. The sperm vector method is a genetic recombination method for introducing an exogenous gene by attaching an exogenous gene into sperm or incorporating an exogenous gene into sperm cells by electroporation, etc. and fertilizing an egg (M. Lavitranoet et al., Cell, 57, 717, 1989).

In vivo site-specific genetic recombination such as cre/loxP recombinase system of bacteriophage P1, FLP recombinase system of *Saccharomyces cerevisiae*, and such, can be used. The method for introducing a transgene of a target protein into nonhuman animals using retrovirus has been reported.

Methods for preparing transgenic animals by microinjection are performed, for example, in the following manner. A transgene basically composed of a promoter regulating expression, a gene

encoding a specific protein, and poly (A) signal is provided. Expression pattern and level for all lineages should be confirmed since the expression pattern and level of a specific molecule depend on the promoter activity, and prepared transgenic animals vary among lineages depending on the number of copies and introduction site on chromosomes of an introduced transgene. A sequence of introns to be spliced at upstream of poly (A) signal may be introduced when the expression level is known to vary depending on noncoding region and splicing. It is important to use a gene as pure as possible for introducing into a fertilized egg. An animal to be used includes a mouse for collecting fertilized eggs (5 to 6 weeks old), male mouse for crossing, pseudopregnant female mouse, vasoligated male mouse, etc.

To efficiently obtain fertilized eggs, ovulation can be induced by gonadotropin, etc. A fertilized egg is collected, and a gene is injected into a male pronucleus of the egg by microinjection using an injection pipette. Animals for returning the treated eggs into an oviduct are prepared (pseudopregnant female mice, etc.), and about 10 to 15 eggs are transplanted per each individual. Introduction of the transgene into a new-born mouse is confirmed by extracting genomic DNA from the tip of the tail and detecting the transgene by Southern hybridization or PCR methods, or by the positive cloning method in which a marker gene that is activated only upon homologous recombination is inserted. Expression of the transgene can be confirmed by detecting a transgene-derived transcript by Northern hybridization or RT-PCR methods. Detection by Western blotting method is also possible using an antibody specific to a protein.

A knockout mouse of the present invention is prepared so as to lose the function of mouse Meg-1 gene. A knockout mouse means a transgenic mouse in which a certain gene is destroyed by homologous recombination technology to eliminate its function. A knockout mouse can be prepared by conducting homologous recombination using ES cells and selecting ES cells in which one allele is modified and destroyed. For example, genetically manipulated ES cells are injected into a blastocyst or an 8-cell embryo of a fertilized egg to obtain a chimeric mouse having both cells derived from ES cells and from embryo. A

heterozygous mouse in which all of one allele is modified and destroyed can be prepared by crossing a chimeric mouse (chimera means an individual composed of somatic cells derived from two or more fertilized eggs) and a normal mouse. Crossing of heterozygous mice with each other can produce homozygous mice. A transgenic animal of the present invention includes both heterozygotes and homozygotes.

Homologous recombination means the recombination occurring between two genes whose nucleotide sequences are the same or extremely similar through mechanism of genetic recombination. Cells with homologous recombination can be selected by PCR. Homologous recombination can be confirmed by performing PCR using as primers sequences of a part of a gene to be inserted and a part of a chromosomal region into which the gene is expectedly inserted and detecting cells producing amplified products. Homologous recombination in the genes expressed in ES cells can be easily screened by known methods or their modified methods, for example, by binding neomycin resistant gene to the introduced gene to make the cells neomycin resistant after the introduction.

Brief Description of the Drawings

Figure 1 is a micrograph (magnification 40) showing the result of the *in situ* hybridization with Meg-1 specific probe in the normal human glomerulus tissue.

Figure 2 is a micrograph showing the result of the *in situ* hybridization with Meg-1 specific probe in the normal human glomerulus tissue at the magnification higher than that of Figure 1 (magnification 80).

Figure 3 is a photograph showing the result of the electrophoresis of total mRNA prepared from rat glomerulus in which mesangial nephritis was induced by anti-Thy1 antibody (represented by "total RNA" in the figure), and a photograph showing the result of Northern blot analysis using Meg-1 specific probe (represented by "rat Meg-1" in the figure).

Best Mode for Carrying out the Invention

The present invention is illustrated in detail below with

references to examples, but is not to be construed as being limited thereto.

Example 1: Primary culture of human mesangial cells

Human glomerular renal mesangial cells were isolated from the normal human kidney excised from a 58 year-old male. Renal cortex was separated under the sterilized condition, minced, and passed through several sieves. Pore diameters of the used sieves were decreased stepwise, and the trapped glomerulus by the sieve at the pore diameter of 75 to 200 μm was washed and incubated with 100 μg/ml collagenase (Washington Biochemical) at 37°C for 20 min. After washing, the glomerulus was resuspended in medium 199 (Gibco BRL, Gaithersburg, MD) containing 25 mM Hepes, 10% Nu-serum (Collaborative Biomedical Products, Bedford, MA), and antibiotics (10 μg/ml of penicillin, streptomycin, and fungizone), and incubated in the 5% CO_2 incubator. At the third passage, mesangial cells were identified based on a series of criteria such as typical morphological characteristics, resistance to trypsin, puromycin, and D-valine, positiveness against immunostaining of actin (Zymed Laboratories, San Francisco, CA), anti-very late antigen (VLA)-1, 3, 5 (Immunotech), and negativeness against immunostaining of VIII factor (Dako, CA).

Example 2: Isolation of mRNA from human cultured mesangial cells

At the sixth passage, total RNA was isolated from human mesangial cells using guanidine isothiocyanate (GTC) method. The confluent culture of the mesangial cells in the medium containing serum of the cells of Example 1 was washed with phosphate buffer saline (PBS), and dissolved in 5.5 mM GTC solution. DNA was removed by passing through an 18-gauge needle. Nuclei and other cell debris were precipitated by centrifugation at 5,000X g for 90 sec. Supernatant was carefully loaded on the layer of cesium trifluoroacetate (CsTFA) and centrifuged at 125,000X g at 15°C for 24 hours. RNA pellet was dissolved in TE buffer. Poly$(A)^+$ RNA was isolated using oligo dT cellulose column (Pharmacia).

Example 3: Construction of 3'-directed cDNA library

cDNA was synthesized using the vector primer based on pUC19 (Norrander J. et al., Gene, 26, 101-106, 1983) with poly(A)$^+$ RNA as a template. This vector primer DNA comprised the HincII end and the PstI end with a T tale, and dam-methylated at the MboI site (GATC). After synthesizing the second strand, the cDNA sequence and the single BamHI site in LacZ gene of the vector were digested with MboI and BamHI, respectively, and circularization and ligation were conducted at the low DNA concentration. A portion of the ligation mixture was transformed to *E. coli*. The obtained transformants were randomly selected and individually dissolved by simply heating. The inserted sequence of cDNA was amplified by the paired PCR using primers (5'-TGTAAAACGACGGCCAGT-3'/SEQ ID NO: 3 and 5'-ACCATGATTACGCCAAGCTTG-3'/SEQ ID NO: 4) flanking the pUC19 cloning site. The obtained short double stranded DNA was used for the cycle sequence determination reaction and analyzed by an automatic sequencer.

Example 4: Isolation of genes expressed specifically in mesangial cells

In order to identify genes expressed specifically in mesangial cells, the present inventor conducted large scale DNA sequencing and data processing by computers. Transcripts in the various different cells and organs could be simultaneously compared (Y. Yasuda et al., Kidney International 53:154-158, 1998; K. Matsubara et al., Gene. 135, 265 (1993); K. Okubo et al., Nat. Gen. 2, 173 (1992)). Large scale DNA sequencing of the 3'-domain cDNA library of human cultured mesangial cells was conducted, and randomly selected 1836 clones were sequenced for their partial sequences. The sequence homology among clones was mutually compared, and further compared with that in DNA data bank GenBank using FASTA program. mRNA from various organs and cells were analyzed using dot blot to select clones expressed specifically in mesangial cells. As a result, some clones detected at extremely high frequency in the mesangial cell cDNA library were obtained.

Example 5: Cloning of the full-length cDNA by 5'RACE

The following experiment was carried out using "5'-Full RACE Core Set" (Takara shuzo, Co. Ltd.). To a 0.5 mL microtube were added 4.0 μL of human poly(A)$^+$ RNA prepared from human mesangial cell culture (0.5 μg/μL), 1.5 μL of 10X RT buffer, 0.5 μL of RNase inhibitor (40 U/μL), 1 μL of AMV Reverse Transcriptase XL (5U/μL) and 1 μL of a 5'-terminal phosphorylated RT primer (5'-pTCAGAGAGGTCATTC/SEQ ID NO: 5, 200 pmol/μL), and the total volume was adjusted to 15 μL with 7 μL of RNase free dH_2O. The reaction mixture was placed in the "Takara PCR Thermal Cycler" (Takara shuzo, Co. Ltd.) and incubated for 10 minutes at 30°C, for 60 minutes at 50°C, for 2 minutes at 80°C and for 10 minutes at 4°C to obtain the first-strand cDNA.

Fifteen microliters of the reaction solution was added to a 0.5 mL microtube containing 15 μL of 5X hybrid RNA denaturation buffer and 45 μL of H_2O. After adding 1 μL of RNaseH, a reaction was conducted for one hour at 30°C. After the reaction, 150 μL of ethanol was added to the reaction mixture, which was then cooled for 30 minutes at -70°C. The mixture was centrifuged, and the supernatant was removed to collect the pellet.

To the pellet, 8 μL of 5X RNA (ssRNA) ligation buffer, 20 μL of 40% PEG #600 and 12 μL of H_2O were added and mixed well. One microliter of T4 ligase was then added to the mixture, which was then incubated for 15 hours at 16°C to obtain cyclized single-stranded cDNA.

The cyclized single-stranded cDNA thus obtained was used as template after 1:10 dilution with TE buffer, followed by primary PCR. The reaction conditions were 5 μL of 10X LA PCR buffer II (Mg^{2+}plus), 8 μL of dNTP mixture (2.5 mM), 0.5 μL of primary PCR S1 primer (5'-TCATTGATGGGTCCTCAA/SEQ ID NO: 6, 20 pmol/μL), 0.5 μL of primary PCR A1 primer (5'-AGATTCTTGAGCTCAGAT/SEQ ID NO: 7, 20 pmol/μL), 0.5 μL of TaKaRa LA TaqTM (5U/μL), and the total volume of the mixture was adjusted to 50 μL with sterilized water. The mixture was placed in the "Takara PCR Thermal Cycler", heated for 3 minutes at 94°C, and subjected to 25 cycles of the reaction as follows: for 30 seconds at 94°C, for 30 seconds at 60°C and for 2 minutes at 72°C.

One microliter of the primary reaction solution was used as template, to which 5 μL of 10X LA PCR TM buffer II (Mg^{2+}plus), 8 μL

of dNTP mixture (2.5 mM), 0.5 μL of the secondary PCR S2 primer 5'-AATGGTGGCATAAACATG/ SEQ ID NO: 8, 20 pmol/μL), 0.5 μL of the secondary PCR A2 primer 5'-ACAGACAAATTGAACTTC/ SEQ ID NO:9, 20 pmol/μL), and 0.5 μL of TaKaRa LA TaqTM (5U/μL) were added, and the total volume of the mixture was adjusted to 50 μL with sterilized water. The mixture was placed in the "Takara PCR Thermal Cycler", and subjected to 30 cycles of the reaction for 30 seconds at 94°C, for 30 seconds at 60°C and for 2 minutes at 72°C.

After the appearance of the band was verified by 0.75% agarose gel electrophoresis, 1 μL of the reaction solution was subcloned using the "Original TA cloning Kit" (Invitrogen). The nucleotide sequence of the gene fragment inserted into the plasmid recovered was determined by the dideoxy termination method.

The result showed that the nucleotide sequence obtained contained the region encoding the N-terminal portion of the gene product and about 50 additional nucleotides as the 5'-untranslated region. The predicted position of the initiation codon ATG corresponded to that in the consensus sequence, giving the longest ORF (fulfilling "the first ATG rule"). The amino acid sequence consisting of 950 amino acid residues based on the longest ORF found in the cDNA sequence was determined as the deduced amino acid sequence of the gene product. The protein having this amino acid sequence was designated Meg-1 by the present inventor. The nucleotide sequence of the meg-1 cDNA is shown in SEQ ID NO: 1 and the deduced amino acid sequence thereof are shown in SEQ ID NO: 2.

Example 6: Functional analysis of the mesangium-specific gene (1)

Amino acid homology search of the SwissProt database with FASTA program confirmed that Meg-1 is a novel protein. It also verified that protein serine/threonine phosphatase 4 related protein $PP4_{R1}$ shows a homology. Meg-1 has 97.9% homology to $PP4_{R1}$ in the amino acid sequence and 98.3% in the nucleic acid sequence of cDNA. In addition, a nucleotide sequence for a portion of the amino acid sequence shown as SEQ ID NO:2 (173 amino acid residues extending positions 778 to 950) is 99.4% identical to the nucleotide sequence (HSU79267) of a cDNA submitted as an EST derived from fetal brain EST, and 100%

identical in the amino acid sequence. This clone is derived from Soares library 1NIB from IMAGE consortium, and the EST is merely a partially determined sequence whose function and bodymap is totally unknown.

Subsequently, a motif search was conducted on the amino acid sequence of Meg-1. PSORT WWW Server (http://psort.nibb.ac.jp:8800/) and MOTIF (http://www.motif.genome.ad.jp/) were used for the search. The result revealed that Meg-1 contains several phosphorylation motifs. Specifically, the presence of the motifs for phosphorylation by the kinases shown below has been identified. Additionally, six N-glycosylation sites were verified. Furthermore, the presence of a nuclear localization signal was also suggested. The nuclear localization was predicted to be 52.2%.

cAMP/cGMP dependent protein phosphorylation site: 1
casein kinase II phosphorylation site: 22
protein kinase C phosphorylation site: 11
tyrosine kinase phosphorylation site: 2

Furthermore, based on these facts, it was confirmed that the above-predicted amino acid sequence with 950 amino acid residues corresponds to the amino acid sequence of Meg-1. The theoretical pI of the protein with this amino acid sequence is 4.49.

Example 7: Functional analysis of Meg-1 (2) - tissue distribution

Northern blot analysis of Meg-1 was performed as described in the following. The positive clone insert of the 3' directed cDNA library (Example 3) was labeled with RI by random DNA labeling and was used as a probe. Poly $(A)^+$RNA (2 μg) isolated from the below-mentioned cells to be used as samples was separated on a 1% agarose gel containing 2.2 M formamide and was transferred onto a nitrocellulose filter. The filter was hybridized in Rapid Hyb solution (Amersham, Arlington Heights, IL). After hybridization, it was washed with final stringency of 0.1X SSPE/0.1% SDS at 60°C.

Samples for Northern blot analyses of multiple human primary culture cells and tissues, and for Northern blot analysis of human cancer cell lines were purchased from Clontech (Palo Alto, CA). As samples of primary culture cells, 2 μg of poly $(A)^+$RNA from primary

culture cells of mesangial cells, human dermal epithelial cells, human renal cortical epithelial cells, human endothelial cells of the umbilical vein, and human smooth muscle cells were used. For Northern blot analysis of human cancer cell lines, 2 μg of poly $(A)^+$RNA derived from promyelocytic leukemia HL-60, HeLa cells S3, chronic myeloid leukemia K-562, lymphoblastic leukemia MOLT-4, Burkitt's lymphoma Raji, adenocarcinoma of the large intestine SW480, lung cancer A549, and melanoma G361 were used as samples. For Northern blot analysis of the tissues, 2 μg of poly $(A)^+$RNA derived from the heart, brain, placenta, lung, liver, skeletal muscles, kidney and pancreas were used as samples. Hybridization and washing were carried out as described above. The results are as indicated in Tables 1 to 3.

Table 1

Primary culture cells	
Human mesangial cells	+ + +
Human dermal fibroblast cells	+ +
Human renal cortical epithelial cells	+
Human endothelial cells of the umbilical vein	± to +
Human smooth muscle cells	± to +

Table 2

Human cancer cell lines	
Promyelocytic leukemia HL-60	+ +
HeLa cells S3	+ + +
Chronic myeloid leukemia K-562	+ + +
Lymphoblastic leukemia MOLT-4	+ + +
Burkitt's lymphoma Raji	– to ±
Adenocarcinoma of the large intestine SW480	+ + +
Lung cancer A549	+ + +
Melanoma G361	+ +

Table 3

Human tissues	
Heart	+ + +
Brain	+ +
Placenta	+ + +
Lungs	−
Liver	+
Skeletal muscle	+ +
Kidney	+ +
Pancreas	+ +

In the Northern blot analysis using Meg-1 cDNA as a probe, a single transcription product (approximately 4.5 kb) was found in the cultured mesangial cells. One of the characteristics in the human primary cell culture was that the Meg-1 gene is especially highly expressed in mesangial cells. In other primary cell cultures, expression in renal cortical epithelial cell and dermal fibroblast are observed, and slight expression in endothelial cell of the umbilical vein and smooth muscle cells was observed. Furthermore, when those in tissues are compared, high expression of Meg-1 was observed in the heart and placenta followed by the expression in the brain, skeletal muscle, kidney, and pancreas. Further, expression in the liver was weak, and no expression could be detected in the lung. Among cultured cancer cell lines, strong expression was observed in HeLa cells S3, chronic myeloid leukemia K-562, lymphoblastic leukemia MOLT-4, adenocarcinoma of the large intestine SW480, and lung cancer A549. It was also expressed in promyelocytic leukemia HL-60 and melanoma G361. Almost no expression was observed in Burkitt's lymphoma Raji.

Example 8: Functional analysis of a Meg-1 (3) — *in situ* hybridization

Meg-1 mRNA expression was evaluated in a tissue by *in situ* hybridization using human renal tissue obtained from the kidney of a normal healthy person. *In situ* hybridization was conducted following known methods (Kidney Int. 52:111 (1997)). Nucleotide

sequence corresponding to position 2879 to 2912 of the human Meg-1 (SEQ ID NO: 10) was used as the probe. As shown in Figures 1 and 2, it was confirmed that cells in the mesangium region of glomeruli are Meg-1 mRNA positive.

Example 9: Rat Meg-1

Acquisition of a rat homologue of Meg-1 was attempted.

Total RNA was collected from rat kidney mesangial cells and cDNA was synthesized. Using this cDNA as template, PCR was performed using various degenerative primers based on the nucleotide sequence of human Meg-1. The degenerative primers were designed in the portions corresponding to the following region of human Meg-1:

Sense strand: nucleotide positions 1-18;

Anti-sense strand: nucleotide positions 2852-2872.

An amplified product with the predicted size was cloned, and its nucleotide sequence was determined. Furthermore, the 3' terminus was partially sequenced using the marathorn method (the method used for isolation and identification of megsin). The nucleotide sequence obtained is shown in SEQ ID NO: 11, and the deduced amino acid sequence is shown in SEQ ID NO: 12.

As a result, a rat Meg-1 homologue whose nucleotide sequence has not been reported so far was isolated. Rat Meg-1 shares a highly conserved structure with human Meg-1, 74.3% at the gene level and 86.3% at the protein level.

In addition, localization of rat Meg-1 in the rat cell and tissue was analyzed using the same method as described in Example 7 with the exception of the sample and probes used. The results were as follows:

Brain	+
Lung	+++
Heart	+
Liver	+
Spleen	++
Kidney	++
Smooth muscle	- to +
Mesangial cells	+++

Renal epithelial cells - to +

Renal endothelial cells - to +

Since Meg-1 is highly expressed in the kidney (mesangial cells in particular) over the species including both humans and rats, it is possibly one of the functional genes that are involved in the mesangial cell function.

Example 10: Specific expression of Meg-1 in the mesangial nephritis-induced glomerulus

It is known that mesangial nephritis can be induced in rat by administering a monoclonal antibody raised against the rat Thy1 antigen (Yagi M, Yamamoto T, Nagano N, Kato S, Kusaka M, Kawasaki K, Yaoita E, Kihara T: Transient expression of type I collagen in glomeruli with anti-Thy-1 antibody-induced mesangial proliferative lesions. Pathol Int 45: 409-414, 1995). By this method, mesangial nephritis was induced in the rat glomerulus, and Meg-1 expression was detected there using Northern blot analysis.

Anti-rat Thy1 monoclonal antibody was prepared by using a hybridoma line producing the antibody (purchased from European Collection of Animal Cell Cultures (Sulisbury, UK)) (Salant DJ, Darby C, Couser WG: Experimental membranous glomerulonephritis in rats. J Clin Invest 66: 71-81, 1980). In brief, the hybridoma cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum, and 1x 10^6 cells suspended in 0.5 ml of RPMI-1640 were inoculated into the peritoneal cavities of 10-week-old male Balb/c mice which were injected with incomplete Freund's adjuvant intraperitoneally 7 days earlier. Within 2 weeks after the hybridoma injection, ascites was obtained from the mice. The ascitic fluid was centrifuged to remove insoluble materials, and the supernatant was pooled. Then, the pooled ascites was dialyzed against PBS, and the IgG-rich fraction was precipitated with 50% saturated ammonium sulfate. The crude antibodies were applied to HiTrap Protein A column (Pharmacia Biotech, Tokyo, Japan) to purify IgG. IgG adsorbed on the column was eluted into the elution buffer (0.1 M sodium citrate, pH 5), and was dialyzed against PBS before use.

Meg-1 mRNA expression in rat glomeruli where mesangial

nephritis was induced was detected as follows. All animal experimentation was conducted in accord with Guide for Animal Experimentation, Faculty of Medicine, University of Tokyo. After male Wistar rats, 6 weeks old (purchased from Charles River Japan (Yokohama, Japan)) were bred for 1 week, they received a 1.2 mg/Kg body wt intravenous injection of IgG1 mouse monoclonal anti-Thy1 antibodies (OX7) or vehicle (control). Before the administration, and 2, 4, 7, 14, and 28 days after the administration, kidneys were excised from six respective rats and cut into small pieces. Glomeruli were separated from the small pieces through a sieve. Total RNA was isolated from these glomeruli and subjected to Northern blot analysis. A fragment having the sequence of 841 to 1979th nucleotide (SEQ ID NO: 13) of rat Meg-1 was used as the probe. This region is suited for a probe because of low homology to subunit A (regulatory subunit) of protein phosphatase 2A (PP2A). The probe was prepared by subcloning a fragment that was amplified by PCR using rat mesangial cell cDNA as the template into pGEM-T easy and digesting it by EcoRI.

The result of the Northern blot analysis confirmed that Meg-1 expression remarkably increases in the glomeruli where mesangial nephritis was induced by anti-Thy1 antibody (Figure 3).

Industrial Applicability

The present invention provides a DNA expressed at high frequency in mesangial cells, a protein encoded by the DNA, an antibody binding to the protein, etc. These are supposed to be closely related to mesangial cell-specific bioactivity, and useful for, for example, identifying mesangial cells, and detecting abnormalities in mesangial cells. Moreover, this protein would be helpful for clarifying the functions of mesangial cells and in turn, for investigating causes of diseases relating to mesangial cells. This invention is expectedly applicable to the treatment and diagnosis, and such of diseases relating to mesangial cells, such as mesangial nephritis.

Specifically, the onset or progress of glomerulonephritis may be regulated, for example, by artificially adjusting Meg-1. Alternatively, quantification of mRNA and protein of Meg-1 in the body fluid and mesangial cells may enable diagnosis of renal diseases

such as glomerulonephritis. In glomerulonephritis, functional abnormality is seen in the mesangium region, causing proliferation of mesangial cells or acceleration of matrix production from the cells. The possibility that Meg-1 is involved in these diseases is great.

Meg-1 of this invention and MEGSIN, which was previously reported by the present inventor, share common characteristics with regard to high levels of expression in the mesangial cells. However, the possibility that Meg-1 of this invention is a nuclear protein having nuclear localization signal is suggested because of a homology to a phosphatase, whereas MEGSIN is a protein that is homologous to the SERPIN superfamily, which is a protease inhibitor. In addition, the observation that Meg-1 of this invention is expressed in a variety of tissues is different from the characteristic of MEGSIN, which is expressed specifically in mesangial cells. Therefore, Meg-1 of this invention may possibly be an important protein that supports the function of mesangial cells. This invention, which has elucidated the existence of such an important protein, has great significance.